A novel use of rapamycin and structural analogues thereof

The present invention relates to the use of rapamycin and its structural analogues for the therapeutic treatment of beta-thalassaemia.

The existence of substances capable of inducing the expression of the gene for gamma-globin and the biosynthesis of foetal haemoglobin (HbF) in adult subjects has been known for some time (1). In the majority of cases, those substances are also capable of activating or potentiating the transcription of genes for embryonal and foetal globins in experimental model systems.
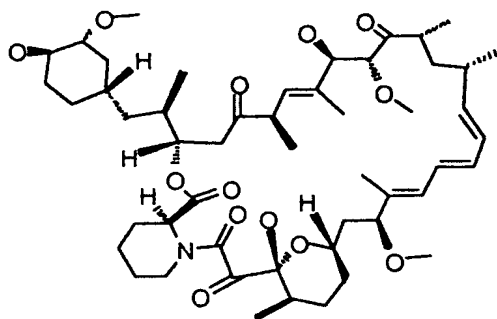
Recently, for example, numerous DNA-binding molecules have been described that have the capacity to bring about an increase in the expression of genes for gamma-globin (2). Among these there may be mentioned cisplatin and analogues of cisplatin, mithramycin and chromomycin, tallimustine (3). Those molecules are efficient modulators of the expression of genes for gamma-globin.

In human beings, activation of the transcription of genes for gamma-globins in adult subjects leads to the production of foetal haemoglobin mimicking the phenotype HPFH (High Persistence of Foetal Haemoglobin) which confers a favourable clinical picture on patients suffering from beta-thalassaemia also in homozygotic form (4). A therapy providing for the use of those molecules in the treatment of patients suffering from beta-thalassaemia could therefore make those subjects less dependent on transfusion therapy (5).

The present invention is based on the need for novel modifiers of the transcription process that can be used in the treatment of beta-thalassaemia and that have a high level of

induction of the expression of gamma-globin genes and a low level of cytotoxicity.

We have surprisingly found that rapamycin, an antibiotic which is described in the prior art and the structural formula of which is illustrated hereinafter, satisfies those requirements.

RAPAMYCIN

In particular, it has been found that rapamycin and its structural analogues are capable of activating the expression of the gene for human gamma-globin.

Rapamycin is an antibiotic produced by *Streptomyces hygroscopicus* and originally described as an anti-fungal agent (6).

The chemical synthesis of rapamycin and its structural analogues - for example 7-(N-hydroxy)-acyl, -carbamoyl and -ureide analogues - has been described by various research groups (see, for example, the bibliographical references 7 and 8).

Rapamycin is currently the active ingredient of drugs (Si-rolimus, Rapamune, antibiotic AY22989) used as anti-inflammatory agents (9, 10) in the treatment of atopical dermatitis (9), allergic contact dermatitis (9) and psoriasis (10), as anti-tumour agents (11, 12) and anti-fungal agents (6). In addition, rapamycin is widely used in therapy as a drug capable of retarding the rejection process (13) in heart (14), kidney (15) and liver (16) transplants.

However, the capacity of rapamycin and its structural analogues to activate the expression of the human gamma-globin gene has not been described hitherto, nor this activity is attributable to its known effects indicated above.

A first subject of the present invention is therefore the use of rapamycin and its structural analogues for the preparation of a medicament for the therapeutic treatment of beta-thalassaemia.

As has been recently described (17, 18), a combined treatment with various modifiers of the transcription process would also permit a further increase in the expression of genes for gamma-globin.

Therefore, a second subject of the present invention is the use of rapamycin or its structural analogues in combination with at least one further modifier of the transcription process for the preparation of a medicament for the treatment of beta-thalassaemia.

According to a preferred embodiment, said further modifier of the transcription process is selected from the group consisting of cytosine arabinoside, retinoic acid, plicamycin, hydroxyurea, guanine, guanosine triphosphate (GTP), guanosine diphosphate (GDP) and guanosine monophosphate (GMP); of

these, cytosine arabinoside and retinoic acid are more pre-
ferred.

The activity of rapamycin as an inducer of erythroid cell
differentiation and the production of mRNA for gamma-globin
was evaluated in human cell cultures. The results of this
study are illustrated in the Tables given in the section re-
lating to the Examples. The data obtained indicate that the
activity of rapamycin is greater than that of reference drugs
(for example, hydroxyurea in the induction of HbF); it has
also been ascertained that the cytotoxic effect which may be
encountered is much lower than that of hydroxyurea.

The Examples which follow are provided for the purposes of
illustration and are not intended to limit the scope of the
invention in any way.

Example 1

The biological activity of rapamycin was evaluated by examin-
ing the capacity of that compound to modulate the expression
of genes for gamma-globin in the human cell line K562, which
is capable of undergoing erythroid differentiation by ex-
pressing genes for gamma-globin if subjected to treatment
with modifiers of the biological response that are suitable
for the purpose (3, 17, 18). The level of differentiation was
evaluated by analysing the positive reaction of the cells to
benzidine (3). Some of the data obtained are given in Table
1, which shows the results of six independent experiments
(mean ± SD). As will be readily appreciated, rapamycin is ca-
pable of bringing about an increase in the percentage of K562
cells that react positively to benzidine (40-45% in the
treated cells, compared with 4.1% of the control K562 cells).
The production of haemoglobin was evaluated by electrophore-
sis on cellulose acetate and by staining the gel with a solu-

tion based on benzidine/H_2O_2 (3). The chief haemoglobin pro-
duced by K562 cells treated with rapamycin was Hb Portland
(alpha2gamma2). The expression of genes coding for gamma-
globins was evaluated by quantitative RT-PCR (reverse tran-
scriptase PCR) (3). The data obtained demonstrate an increase
in the intracytoplasmic accumulation of mRNA for gamma-
globin. These evaluations were carried out after 6 days of
induction with 20 nM rapamycin. The results of the quantita-
tive RT-PCR are shown in Table 2.

Table 1

Compound	Concentration (nM)	[a]Erythroid differentiation (%)
-	-	4.1±3.7
Rapamycin	20	45.5±5.8

[a] Erythroid differentiation = percentage of K562 cells that
react positively to benzidine (mean ± SD of 6 experiments).

Table 2

Compound	Concentration (nM)	[b]mRNA for gamma-globin
-	-	1
Rapamycin	20	2.4

[b] The accumulation of RNA for gamma-globin is given in the
Table as an increase compared with that of untreated control
K562 cells. The technique used was that of quantitative RT-
PCR (21, 22) using the following primer and probe oligonu-

cleotides: gamma-globin forward primer, 5'-TGG CAA GAA GGT
GCT GAC TTC-3'; gamma-globin reverse primer, 5'-TCA CTC AGC
TGG GCA AAG G-3'; gamma-globin probe 5'-FAM-TGG GAG ATG CCA
TAA AGC ACC TGG-TAMRA-3' (FAM = 6-carboxy fluorescein, TAMRA
= 6-carboxy-N,N,N',N'-tetramethylrhodamine).

Example 2

In order to check whether rapamycin was capable, in addition
to inducing K562 cell differentiation, of stimulating the
production of mRNA for gamma-globin in human erythroid pre-
cursors isolated from peripheral blood, the technique pub-
lished by Fibach et al. (19, 20) was used. This technique
provides for two stages: in the first stage, the cells iso-
lated from peripheral blood of a subject who is healthy or
suffering from a haemopoietic pathology, such as sickle cell
anaemia or beta-thalassaemia, are sown in a culture medium to
which 10% of conditioned medium derived from the vesicle car-
cinoma cell line 5637 has been added. The second stage con-
sists in cultivating the isolated cells in a suitable culture
medium, supplemented by the erythropoietin hormone, 30% foe-
tal bovine serum, 2-mercaptoethanol, albumin, glutamine and
desamethasone in order to permit the proliferation and matur-
ing of stem cells of the erythroid type. In this stage the
cells can be treated with potential HbF inducers.

For example, in this system it was demonstrated that hydroxy-
urea, an inhibitor of DNA synthesis currently used in the ex-
perimental therapy of beta-thalassaemia, is capable of bring-
ing about the production of HbF.

The results obtained with rapamycin demonstrated an increase
in the production of mRNA for gamma-globin in cells treated
with rapamycin compared with the same untreated cells (3.75

times), which increase is greater than that which may be encountered using hydroxyurea (Table 3).

Table 3

Compound	Concentration (µM)	[a]mRNA for gamma-globin
–	–	1
Rapamycin	3	3.73
Hydroxyurea	120	2.25

[a] The accumulation of RNA for gamma-globin is given in the Table as an increase compared with that of untreated control erythroid precursors. The technique used was that of quantitative RT-PCR (21, 22) using the primer and probe oligonucleotides described in Table 2.

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